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ULTRASTRUCTURAL CHANGES AS INDICATORS OF THE EFFECTIVENESS OF A HYAMINE QUATERNARY COMPOUND AS AN ALGACIDE IN CONTROLLING GROWTH OF THE FILAMENTOUS ALGA, CLADOPHORA (CHLOROPHYTA)

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Report Prepared For:

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Water Resources Branch
Ontario Ministry of the Environment

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ABSTRACT

Ultrastructural changes were used to assess the effectiveness of an algicide, Hyamine, in controlling the growth of a filamentous alga, *Cladophora*. At 0.5 mg Hyamine L^{-1}, ultrastructural changes were observed in the apical and intercalary cells but not in the mature vegetative cells. During the deterioration process, large numbers of starch grains were lost, thylakoid membranes were fragmented and vesicles formed in the chloroplast stroma. As the chloroplasts degenerated, apical cells collapsed and thus, halted growth of the filamentous alga temporarily. After an incubation period of 25 days, the sample in the 0.5 mg Hyamine L^{-1} solution showed numerous dead apical cells. Only a few survived. Since quaternary ammonium compounds have been reported to be the least toxic to fish among the many tested algicides, they could be a useful chemical for controlling growth of *Cladophora* during peak growth.

INTRODUCTION

Many organic compounds (e.g., atrazine, colchicine and chloramphenicol) are capable of altering the fine structure of algal cells at low concentrations (Ashton et al. 1966, Ben-Shaul and Ophir 1970, and Smith-Johannsen and Gibbs 1972). Thus, by identifying the sites of inhibition and assessing the amount of damage to the organelles, it is possible to determine the effectiveness of chemical compounds used as algicides to control the growth of certain algal species, particularly those that are difficult to handle in conventional assays, e.g., *Cladophora*.

Cladophora, an ubiquitous nuisance filamentous alga, thrives in many rivers and along lake shorelines (Moore 1978, Neil and Owen 1964, and Shear and Konasewich 1975). Once established, *Cladophora*, requires only low level nutrients to attain its maximum growth rate (Wong and Clark 1976). A rich supply of nutrients thus encourages development of biomass. In fair weather, *Cladophora* proliferates to form large mats within days. It is very difficult to curb its growth through mechanical means especially in waters where silica and vitamins are abundant (Moore and Traquair 1976, Moore and McLarty 1975, Hoffmann 1990, and Hoffmann and Graham 1984).

Among the groups of algicides used, some are selective (e.g., 2, 3-dichloronaphthoquinone is for controlling blue-green algae; Fitzgerald and Skoog 1954), while others are for general use (e.g., Cu and Cl).

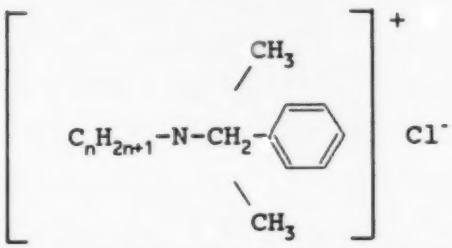
Certain quaternary ammonium compounds, such as dodecyl dimethyl benzyl ammonium chloride (DAC), have been reported to be effective algicides for inhibiting the growth of *Stigeoclonium*, a filamentous green alga (Palmer 1956), but it is not known if DAC produces the same growth inhibitory effect on other attached filaments such as *Cladophora*. The objective of this study, therefore, is to determine the algistatic concentration of Hyamine (a more powerful quaternary ammonium compound) for *Cladophora* cells and to understand the changes taking place at subcellular levels due to effects of the algicide. Algistatic concentration is defined as the lowest inhibiting concentration that allows treated cells to

regrow after they are reintroduced back into fresh growth medium (Fitzgerald 1971, and Payne 1975).

METHODS

An axenic culture of *Cladophora glomerata* (L.) Kutz. (UTCC #13) was obtained from the University of Toronto Culture Collection, Department of Botany. The algal culture was maintained in Bold's basal medium (BBM) supplemented with a vitamin mix (B_{12}, biotin and thiamine, Hutchinson and Stokes 1975) and 5 mg L^{-1} Si as $Na_2Sio_3 \cdot 9H_2O$ (Moore and Traquair 1976).

Hyamine 3500 was purchased from the Rohm and Haas Company. The algicide is an 80% concentrate in ethanol, containing the active ingredient n-alkyl (50% tetradecyl, 40% dodecyl and 10% hexadecyl) dimethyl benzyl ammonium chloride with a chemical structural formula of



The molecular weight of the chemical is 359.6. Stable and non-volatile, it has been recommended by the company (Rohm and Haas 1978) as a disinfectant in swimming pools at low concentrations.

Algal assays were conducted in duplicate in 250 mL Erlenmeyer flasks containing 100 mL BBM solution. The toxicity range of Hyamine for *Cladophora* was tested at the beginning of the study. In the final assays, the Hyamine concentrations were set at 0.3, 0.4, 0.5, 0.8 and 1.0 mg L^{-1}. Zero Hyamine concentration was used as a control. About 0.5 gm (wet weight) of *Cladophora* filament was used as the inoculum. The cultures were incubated for 4 days at 20°C in an environmental chamber where the light and dark cycle was set at 12:12 and the light intensity was 60 μEin m^{-2} sec^{-1}.

On day 4, a small amount of plant material was removed from each concentration for morphological examination using a light microscope. One set of samples was put aside for electron microscopic preparation; the other set was incubated for another 3 weeks for further morphological evaluation. These tests were repeated at the end of the experiment.

For electron microscopy, *Cladophora* filaments were fixed in 4% (cacodylate-buffered) glutaraldehyde for 6 hours and then postfixed in 2% (cacodylate-buffered) osmium tetroxide for 1 hour. The dehydration ethanol series was 30%, 50%, 70%, 90%, 95% and 100%.

For infiltration, the Spurr's medium to ethanol ratios were 1:3, 1:2 and 2:3 v/v. The *Cladophora* filaments were then embedded in 100% Spurr's medium in an aluminum dish. The samples were polymerized at 70°C overnight.

By using a dissecting microscope, the apical cells of the filaments were traced and marked on the embedding material. Small columns of specimens were cut out with a jeweller's saw. Thin sections were prepared with a glass knife on a nicrotome. These sections were then stained with uranyl acetate and lead citrate. Observations were made using a Siemens (ELMISKOP 102) electron microscope.

RESULTS

Figure 1a is a cross-section of a *Cladophora* apical cell. In this young vegetative cell, the greater part of the cell lumen was occupied by the central vacuole. Surrounding this central vacuole was a thin layer of cytoplasm containing many chloroplasts, most of which carried a prominent starch grain. Although *Cladophora* cells are known for their large single chloroplast (Chan et al. 1978 and Prescott 1964), where intertwining thylakoid membranes are found as we observed in a number of field *Cladophora* species, this culture species appeared to have many chloroplasts. First of all, the chloroplast envelopes were not connected (Fig. 1b). Secondly, the 2-disc thylakoid membranes running alongside the starch grains terminated at both ends close to the chloroplast envelope (arrows). Thirdly, the chloroplasts multiplied by constriction of the envelope and finally, these chloroplasts, in the

presence of toxicants, responded and degenerated unevenly and independently of each other as described later in the text. Internal to the circle of chloroplasts in the cytoplasm lay one or sometimes two nuclei. Pyrenoids were found with large starch plates. Groups of osmiophilic bodies (plastoglobuli) were also located in between thylakoid membranes in chloroplasts.

In the presence of Hyamine, after incubation for 4 days in a 0.5 mg L^{-1} solution, the *Cladophora* cells in the filaments showed no abnormality when observed under the light microscope. Internally, the vegetative cells were healthy. Yet in the apical cells (Fig. 2a), a large reduction of starch grains was evident. The thylakoid membranes in two of the chloroplasts appeared in short bundles (pointers). Small vacuoles erupted in the central vacuole, many with receding contents (arrows).

At 0.8 mg Hyamine L^{-1}, the vegetative cells still remained unaffected. The chloroplasts in the apical cells were no longer dividing (Fig. 2b). They had swollen in size, each with disarrayed bundles of thylakoid membranes. In one chloroplast, there were four pyrenoids in a cluster (arrow). In the cell lumen, following the disappearance of the cytoplasmic strands, vacuoles fused into large holes.

Under high power, chloroplasts in the apical cells had deteriorated in stages, even at 0.5 mg Hyamine L^{-1}. First, there was the loss of starch grains, then the loss of the interthylakoidal spaces and the appressed membranes, then the detachment of membranes from the sides and ends of the thylakoid bands (Fig. 3a), followed by the fragmentation of membranes (Fig. 3b) and the formation of vesicles in the stroma (Fig. 3c), and finally, the amassing of thylakoids behind the cells walls before the chloroplasts dispersed throughout the cells (Fig. 3d).

Pyrenoids degenerated in very much the same manner in the chloroplasts. There was a gradual loss of starch plates around the pyrenoids just as the thylakoidal membranes broke into small bundles in the stroma (Figs. 4a and 4b).

Among organelles, nuclei have always been the most resistant fine structure, remaining unaltered even at concentrations as high as 0.8 mg L^{-1} (Fig. 5a). At this concentration, Golgi complexes did not develop; mitochondria were altered by the formation of vesicles (Fig. 5b) and concentric membranes (Fig. 5c), a reduction of cristae and presence of empty matrices (Figs. 5c and 5d).

To assess the prolonged toxic effect of Hyamine on *Cladophora* cells, samples were examined under the light microscope after a 25-day incubation. Cells below 0.5 mg L^{-1} were healthy but with less biomass compared to the control. Cells at 0.5 mg L^{-1} contained many dead apical cells (Fig. 6). The few that survived appeared distorted when compared to healthy, mature vegetative cells. At 0.8 and 1.0 mg L^{-1}, all filaments died.

DISCUSSION

If the purpose of applying algicides is to temporarily stop *Cladophora* growth and allow less competitive algal species to thrive such that a balanced biotic community can be reconstructed (Fitzgerald 1971), then the selection of Hyamine quaternary ammonium compounds may be worth considering, as once suggested by Palmer (1956).

About 35 years ago, Maloney and Palmer (1956) reported that quaternary ammonium compounds were very effective in inhibiting the growth of *Stigeoclonium*, (also an attached green filamentous alga). But since then, no further work has been attempted on other filamentous algae with these compounds even though nuisance vegetation such as *Cladophora* has been spreading to pristine waters, such as Lake Huron (Jackson 1988).

Realizing that traditional assays using weight determinations, cell counts, O_2 or ^{14}C uptakes to determine an algistatic concentration with filamentous algae were not easy to manage, particularly when the recovery of the toxin-exposed algal cells was slow, we opted for an alternative by examining the morphological and ultrastructural changes in algal cells.

In fact, by examining the damage done by the toxins at the subcellular level using an electron microscope, we could determine whether the damage was deep or superficial or whether there was a possibility for the cells to regrow depending on the degree of destruction of the ultrastructure.

With our test *Cladophora* species, fine structural changes occurred at 0.5 mg Hyamine L^{-1}. A few apical cells survived the toxicity, although deteriorated after 25 days of incubation. At 0.8 mg L^{-1}, the cells were badly damaged and there is no possibility of regrowth even when the cells were reintroduced into fresh medium. Our algistatic concentration for Hyamine 3500 was therefore determined to be 0.5 mg L^{-1}. At this algistatic concentration,t he Hyamine toxicity was selective to the apical and intercalary cells but not the mature vegetative cells. The site of destruction was the chloroplasts.

Unlike tests with other organic contaminants, e.g., parathion, dieldrin or cyclohexane (in preparation), chloroplasts exposed to these chemicals showed only slight changes; for example, the plastids might be elongated, thylakoid membranes sometimes buckled, interthylakoidal spaces were reduced and starch grains were definitely lost. However, with Hyamine, the thylakoid membranes fractured and crumpled into small fragments. Hyamine also prevented the chloroplasts from dividing. In addition, it altered the permeability of the chloroplast envelope such that unwanted solutes permeated the stroma in the form of vesicles (Orr and Hess 1982).

In our culture species, the chloroplasts were observed to multiply by fission. Daughter chloroplasts were formed as the chloroplast envelope constricted. A chloroplast envelope damaged by Hyamine has led to the development of large sized plastids (Fig. 2b).

We noticed also that the chloroplasts degenerated in varying degrees within the same cell, possibly because the newly formed daughter chloroplasts were more sensitive to toxins. This unevenness of chloroplast degeneration throughout the cell further supported the observations that the chloroplasts could not be a large single plastid.

Similarly, apical cells being young and immature, were found to be more susceptible to Hyamine than their mature counterparts. In studies with low concentrations of pesticides and hydrocarbons, damage occurred to both apical and mature vegetative cells. But with Hyamine (0.5 mg L^{-1}), only the apical and intercalary cells were affected. Mackie and Preston (1974) and Pearlmutter and Lembi (1988), in their studies of cell wall formation in *Cladophora* species, mentioned the slow deposition of microfibrils and alterations of microfibril orientation which crossed each other at decreasing angles as the cell elongated. Could these newly formed cell walls be more permeable to surface-active agents like Hyamine such that these unwanted solutes could reach young chloroplasts more quickly?

Nonetheless, if at 0.5 mg Hyamine L^{-1}, some apical and intercalary cells could survive the toxicity, then the temporary halt to the branching of the apical cells would make the quaternary ammonium compound an ideal algicide for *Cladophora* species by Fitzgerald's definition (1971).

The manufacturers (Rohm and Haas 1976), in their assays with Hyamine compounds, reported that at 0.5 mg L^{-1}, Hyamine 3500 was lethal to *Nostoc* and not to the other unialgal test species (i.e., *Calothrix parietina, Ankistordesmus falcatus, Chlorella pyrenoidosa, Phormidium* sp. and *Oscillatoria* sp.). Also, in their study of algicides, Maloney and Palmer (1956) found that quaternary ammonium compounds were less toxic to fish (fathead minnow) and a much safer chemical to use than Cu, quinones, rosin amines and organic Zn compounds. Then, perhaps Hyamine at low concentrations could be a useful chemical to control *Cladophora* growth in areas where algicides are considered as a last resort.

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LIST OF FIGURES

Legends for all figures:

AC	apical cell
C	chloroplast
CE	chloroplast envelope
Cr	cristae
CS	chloroplast stroma
CW	cell wall
GB	Golgi bodies
M	mitochondrion
N	nucleus
OB	osmiophilic bodies
P	pyrenoid
S	starch grain
SP	starch plate or sheath
T	thylakoids
V	vacuole
Ve	vesicle

Figure 1a — Cross-section of a control apical *Cladophora* cell. (x 6000).

Figure 1b — An enlarged portion of a *Cladophora* cell. Chloroplasts (C) are separated in the cytoplasm. The thylakoid (T) membranes terminate close to the chloroplast envelope (CE) (arrows) x (25,000).

Figure 2a — Cross-sections of Hyamine treated apical cells - at 0.5 mg Hyamine L^{-1} (x 12,600).

Figure 2b — Cross-sections of Hyamine treated apical cells - at 0.8 mg Hyamine L^{-1}. The arrow points at four pyrenoids clustered inside a giant chloroplast (x 14,000).

Figure 3 — Disruptions of chloroplasts in 0.5 mg Hyamine L^{-1}

3a — Detachment of thylakoid membranes (x 50,000).

3b — Fragmentation of thylakoid membranes (x 25,000).

3c — Vesicle formation (x 20,000); and 0.8 mg Hyamine L^{-1}.

3d — Chloroplasts drift apart (x 6,500).

Figure 4a — The pyrenoid of a control cell (x 45,000).

Figure 4b — A Hyamine treated cell (x 25,000).

Figure 5 — At 0.8 mg L^{-1}, the various stages of mitrochendria deterioration.

5a — The nuclear structure is apparently not affected by Haymine toxicity (x 14,500).

5b — With vesicles and connncentric membranes (x 38,000).

5c — With empty matrices (x 50,000).

5d — With distorted cristae (x 80,000).

Figure 6 — Light micrograph of *Cladophora glomerata* after exposure to 0.5 mg Hyamine L^{-1} for 25 days. The discoloured cells (AP) are dead apical cells and the crooked one (arrow) is a surviving apical cell (x 40).

Fig. 1a

Fig. 1b

Fig. 2a

Fig. 2b

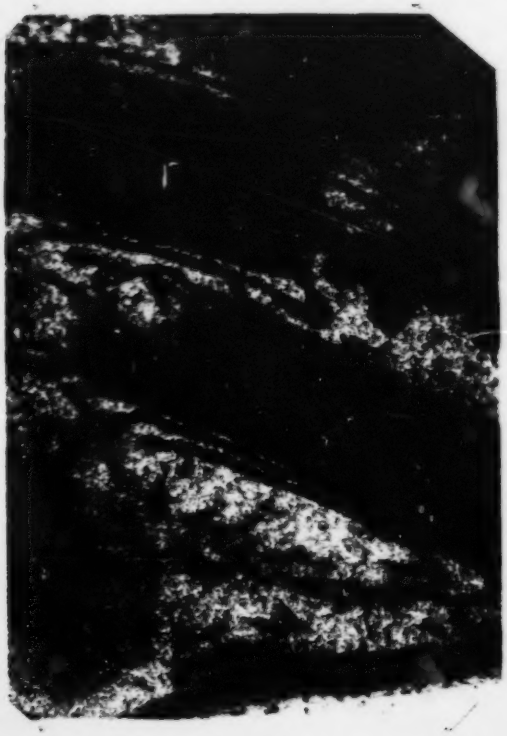

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3d

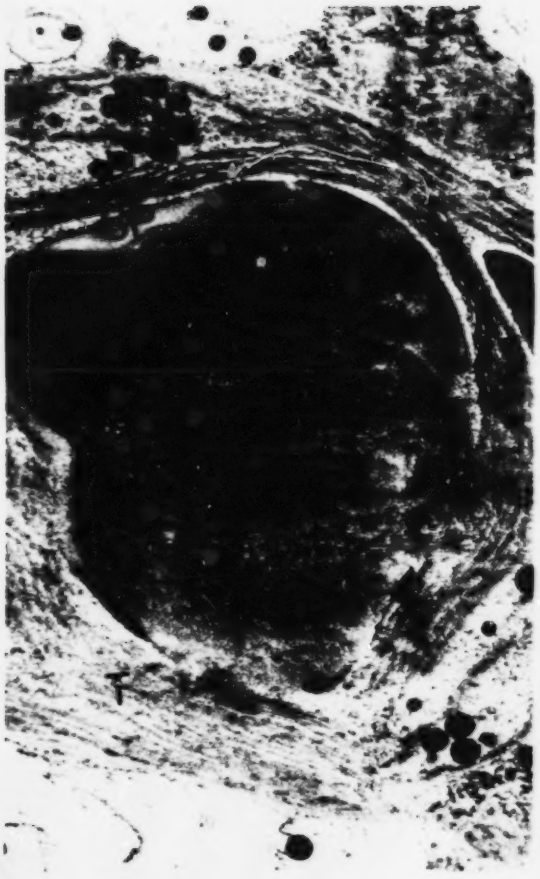

Fig. 4a

Fig. 4b

Fig. 5a

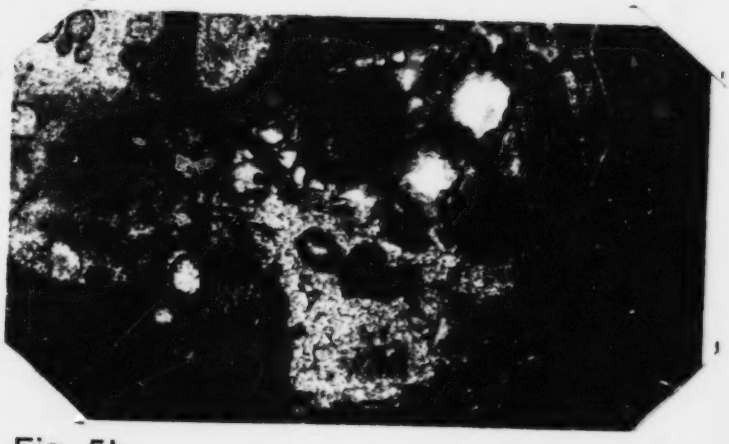

Fig. 5b

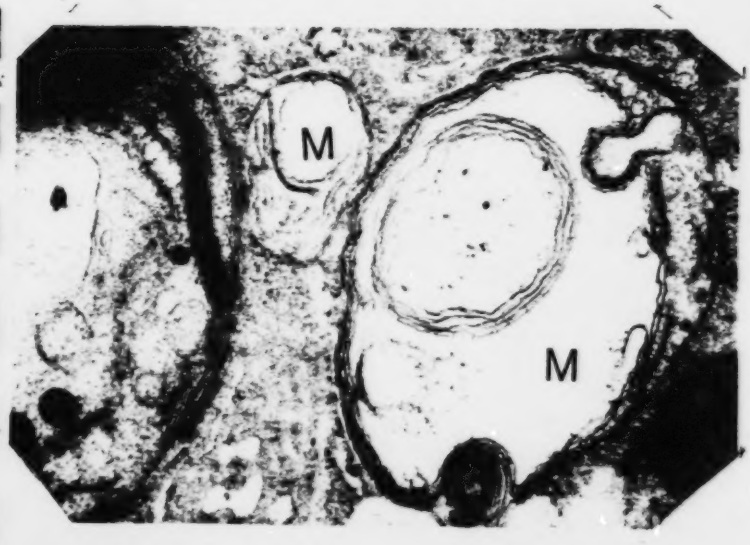

Fig. 5c

Fig. 5d

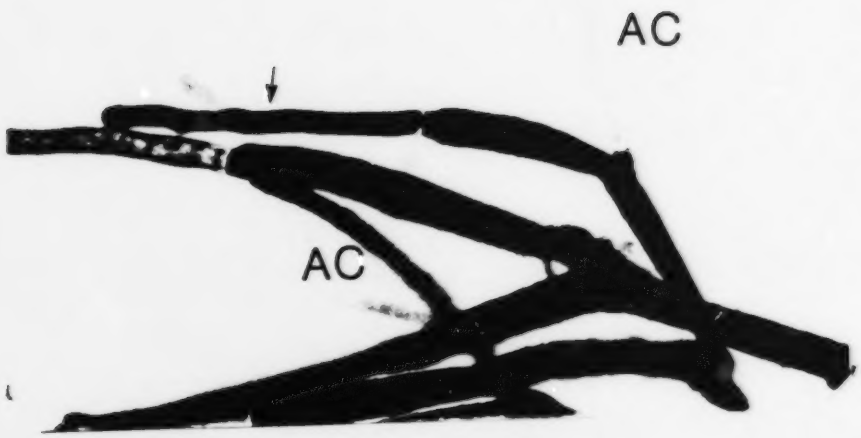

Fig. 6